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Rapid Nocturnal Increase in Ovine Pineal *N*-Acetyltransferase Activity and Melatonin Synthesis: Effects of Cycloheximide

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Abstract: Thirty minutes after the onset of darkness, ovine pineal arylalkylamine *N*-acetyltransferase, *N*-acetylserotonin, and melatonin increase 5- to 10-fold. No significant changes in hydroxyindole-*O*-methyltransferase, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, 5-methoxyindoleacetic acid, and 5-methoxytryptophol are detected at this time. Administration of cycloheximide inhibits the rise in *N*-acetyltransferase and *N*-acetylserotonin, but not melatonin. Unexpectedly, 5-methoxytryptophol increases after cycloheximide treatment. Taken together, these results, although consistent in part with a role for serotonin *N*-acetylation in the regulation of melatonin synthesis in sheep, indicate that an *N*-acetyltransferase-independent mechanism may also be involved. **Key Words:** Ovine—Pineal—Melatonin—*N*-Acetyltransferase—Cycloheximide. **Namboodiri M. A. A. et al.** Rapid nocturnal increase in ovine pineal *N*-acetyltransferase activity and melatonin synthesis: Effects of cycloheximide. *J. Neurochem.* **45**, 832–835 (1985).

On the basis of studies done in the rat, it is now generally believed that the neurally stimulated increase in the activity of pineal *N*-acetyltransferase (NAT; EC 2.3.1.87) is the major factor regulating the nocturnal increase in pineal melatonin synthesis and serum melatonin levels in mammals (Klein and Weller, 1970; Axelrod and Zatz, 1977; Klein et al., 1981). However, recent studies suggest this may not be valid for all species. First, the large nocturnal increase in NAT activity characteristic of the rat is not observed in some species of hamsters or in sheep, even though all show a similar increase in serum melatonin at night (Rudeen et al., 1975; Panke et al., 1978; Goldman et al., 1981; Vanecek and Illnerova, 1982). Second, ovine pineal and serum melatonin levels increase rapidly following a light pulse at night, without any associated increase in NAT activity (Namboodiri et al., 1985).

In the present report we investigated this issue further by studying the effect of cycloheximide on the very rapid nocturnal increase in ovine pineal melatonin and NAT activity (Rollag and Niswender, 1976; Sugden et al., 1985). This approach was used because it is known that cycloheximide blocks the gradual increase in NAT seen in rats; this might not be true for the very rapid nocturnal increase in ovine pineal NAT, which could reflect activation processes. Our results indicate that although the nocturnal increase in NAT is blocked by cycloheximide, the increase in melatonin is not. This suggests to us that both NAT-dependent and NAT-independent mechanisms may be involved in the regulation of melatonin synthesis in sheep.

MATERIALS AND METHODS

Animals

Sheep (12- to 14-month-old Dorset X Rambouillet males, 60–70 kg) were obtained during February and

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Abbreviations used: 5-HIAA, 5-hydroxyindoleacetic acid; HIOMT, hydroxyindole-*O*-methyltransferase; 5-HT, 5-hydroxytryptamine; 5-HTOH, 5-hydroxytryptophol; 5-MIAA, 5-methoxyindoleacetic acid; 5-MTOH, 5-methoxytryptophol; NAS, *N*-acetylserotonin; NAT, arylalkylamine *N*-acetyltransferase.

March from a breeding herd, field-maintained under the supervision of the Division of Intramural Research, National Heart, Lung and Blood Institute. The animals were reared in a natural light environment. Before the experiment they were kept for a 5-day period in a large light-tight box stall with automatically controlled lighting (light/dark 12:12); the dark period started at 1800 hours. The light intensity at the level of the animals was 175–350 lux.

Blood collection and tissue preparation

Blood samples (~10 ml) were removed from the jugular vein at the times indicated; either no light or a dim red light was used at night. Blood was cooled immediately and centrifuged within 1 h. The serum was removed and stored at −30°C. Animals were killed by an intravenous injection of Somlethal (20 ml, Med-Tech, Elwood, KS, U.S.A.). Pineal glands (average wet weight 89.2 ± 7.1 mg) were removed within 2–5 min after death, and frozen immediately in tubes on solid CO_2. Glands were stored (−30°C) until they were homogenized (1 mg wet weight/10 μl) in ammonium acetate (0.01 *M*, pH 6.5) by means of a Polytron homogenizer (3 × 10 s; setting 4; 0–2°C). The crude homogenate was used for all analyses.

Enzyme assays and indole analysis

Pineal NAT and hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4) activities were measured on duplicate samples as described previously (Sugden et al., 1983). Protein was measured using a dye binding method with bovine serum albumin as the standard (Bradford, 1976). The average protein content of the sheep pineal was 48.1 ± 2.7 μg/mg wet weight.

Serum and pineal melatonin were measured in duplicate using a modification (Reppert et al., 1979) of the Rollag and Niswender (1976) radioimmunoassay procedure. A 0.75-ml sample of serum was used, and for pineal melatonin a 20-μl sample of the homogenate was diluted to 8 ml with 0.01 *M* sodium phosphate, pH 7.0, containing sodium chloride (0.9% wt/vol) and duplicate 0.5-ml samples of this were used in the assay.

Pineal indoles were measured using a modification of published HPLC-EC procedures (Mefford and Barchas, 1980). Pineal homogenates (100 μl) were mixed with an equal volume of 0.2 *M* perchloric acid and centrifuged. A 20-μl sample of clear supernatant was loaded onto an Ultrasphere ODS 7.5 × 0.46 cm, 3 μm column (Altex/Beckman, Berkeley, CA, U.S.A.). The running buffer for hydroxyindoles was 0.1 *M* ammonium acetate, 0.1 *M* acetic acid, 50 mg/L EDTA, and 10% acetonitrile and that for methoxyindoles was 0.12 *M* ammonium acetate, 0.05 *M* acetic acid, 50 mg/L EDTA, and 12% acetonitrile. The flow rate (1.0 ml/min) was maintained using a Milton Roy minipump with pulse damping. The detector was an amperometric controller (LC-2A, BAS, West Lafayette, IN, U.S.A.). Eluting hydroxyindoles were oxidized at +0.5 V and methoxyindoles at +0.9 V at a glassy carbon electrode versus a Ag/AgCl reference electrode. The identity of the peaks of interest was verified by altering the pH of the solvent and matching the retention times with those of authentic compounds.

Cycloheximide treatment

Blood samples were taken from 16 sheep at the start of the experiment (1730 hours) and four animals were killed immediately. Six sheep were then injected with cycloheximide (200 mg/sheep i.v. in 10 ml of saline, Sigma, St. Louis, MO, U.S.A.) and six with saline. Blood was collected at 1745 and 1800 hours; lights were turned off at 1800 hours. Further blood samples were taken at 1810, 1820, and 1830 hours. The animals were killed immediately after the last blood sample was taken and their pineal glands rapidly removed and frozen on dry ice.

RESULTS

Serum and pineal melatonin

Serum melatonin levels before lights off were 30–60 pg/ml. The levels increased rapidly after exposure to dark, reaching the nighttime levels (100–150 pg/ml) within 30 min (Fig. 1). Pineal melatonin at this time also increased (7- to 13-fold) (Table 2; Fig. 1), indicating that the increase in serum melatonin

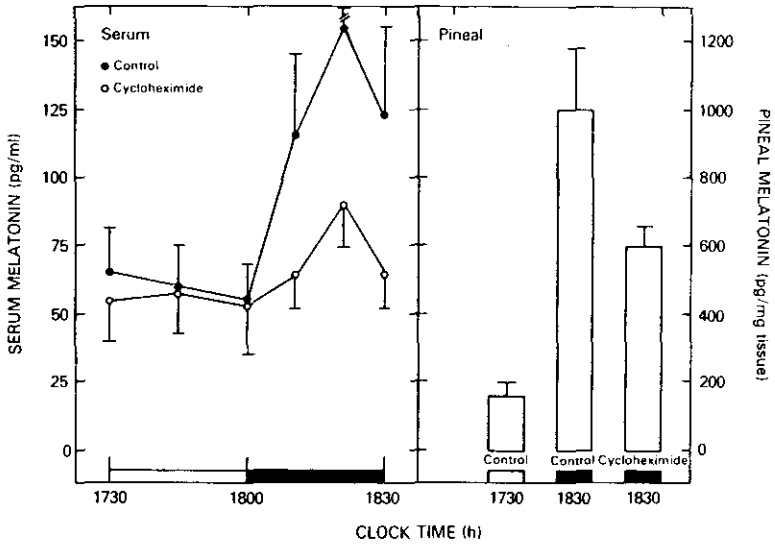

FIG. 1. Effect of cycloheximide on the rapid increase in serum and pineal melatonin at night. Sheep were injected with cycloheximide (200 mg i.v.) or saline after the first blood sample was taken at 1730 hours. Pineal and serum melatonin were measured by radioimmunoassay as described in Materials and Methods. Each point or bar represents the mean ± SEM of six sheep.

was probably caused by an increase in pineal melatonin concentration.

Pineal indoles, NAT, and HIOMT

Pineal NAT activity increased fivefold 30 min after darkness; HIOMT activity did not change (Table 1). *N*-Acetylserotonin (NAS), the immediate precursor of melatonin, increased more than 90-fold, reflecting the increased NAT activity (Table 2). However, 5-hydroxytryptamine (5-HT, serotonin) and its oxidation product, 5-hydroxyindoleacetic acid (5-HIAA), were not significantly changed at this time.

Effect of cycloheximide on melatonin synthesis

Cycloheximide treatment completely prevented the rapid rise in pineal NAT activity, indicating this increase requires protein synthesis (Table 1). Pineal NAS concentration decreased significantly after cycloheximide administration, consistent with the decrease in NAT activity (Table 2). However, cycloheximide treatment did not significantly inhibit the rise in serum and pineal melatonin. Surprisingly, cycloheximide treatment caused a statistically significant increase in pineal 5-methoxytryptophol (5-MTOH) concentration, and tended to increase pineal HIOMT activity and 5-methoxyindoleacetic acid (5-MIAA) concentration.

DISCUSSION

It is clear from this study that the rapid nocturnal rise in ovine serum melatonin is associated with an equally rapid increase in the pineal gland, and that this is caused by an increase in melatonin synthesis. This confirms indications from previous studies (Namboodiri et al., 1985).

The finding of a rapid physiological increase in pineal and circulating melatonin at the start of the night period in the sheep is similar to what has been observed in the monkey (Reppert et al., 1979) but is in sharp contrast to the rat and hamster (Goldman et al., 1981). In the latter species an increase occurs only after a 2–4-h lag period. The mechanisms responsible for these differences in timing are not clear; either the pineal gland or the central timing system regulating the pineal gland could be responsible.

Recent studies indicate that the lag period between lights off and the onset of the NAT increase in rats can be reduced by keeping animals in short nights (Illnerova and Vanecek, 1983), indicating that part of the lag period can be explained by the central timing system. However, there also is evidence available indicating that the lag period is in part explained by the pineal gland. This comes from experiments in which isoproterenol was used to stimulate NAT activity. This approach bypasses the central timing system responsible for stimulating the pineal gland. With isoproterenol a lag period of about 30 min is still observed before any significant increase in NAT occurs. In contrast, within 30 min after the onset of darkness a maximum nocturnal increase occurs in ovine pineal NAT. This indicates that the sheep pineal gland responds to stimulation significantly faster than the rat pineal gland.

One can only speculate on the nature of this difference between the rat and sheep NAT regulatory mechanisms. One possible explanation is that whereas new synthesis of protein, perhaps NAT molecules, is required in both species, new mRNA synthesis is also required in the rat, but not in the sheep, where the required mRNA may already be available.

It is interesting to note that there are no rapid changes in the levels of serotonin and its oxidation products at the onset of darkness, when NAS and melatonin levels increase rapidly. This is in marked contrast to the large reciprocal changes in the levels of 5-HT and its oxidation products at 5 h after the onset of darkness (Namboodiri et al., 1985). Perhaps this occurs because pineal 5-HT stores are slowly depleted by the increased N-acetylation of 5-HT at night and the reduced availability of 5-HT leads to decreases in 5-HT oxidation products. Our current observation that 5-HT oxidation products

TABLE 1. *Rapid changes in pineal enzymes at night and the effect of cycloheximide treatment*

Enzyme	Activity (nmol/min/mg protein)		
	Day	Night	Night + cycloheximide
NAT	0.14 ± 0.07	0.70 ± 0.09[a]	0.12 ± 0.04[b]
HIOMT	0.055 ± 0.006	0.056 ± 0.012	0.091 ± 0.013

All values represent means ± SEM of four to six sheep. Pineal from "day" (1730 hours; untreated), "night" (1830 hours; vehicle-treated), and "night + cycloheximide" (1830 hours; cycloheximide 200 mg i.v.) sheep were obtained as described in Materials and Methods. Pineal NAT and HIOMT levels were measured as described (Sugden et al., 1983).

[a] Significantly different from the "day" group ($p < 0.05$).

[b] Significantly different from the "night" group ($p < 0.05$).

TABLE 2. *Rapid changes in pineal indoles at night and the effect of cycloheximide treatment*

Indole	Concentration (ng/mg wet weight)		
	Day	Night	Night + cycloheximide
5-HT	3.20 ± 0.86	2.10 ± 0.84	3.17 ± 1.07
5-HIAA	19.6 ± 1.8	17.0 ± 1.7	29.0 ± 5.7
5-HTOH	0.24 ± 0.01	0.42 ± 0.05	0.65 ± 0.12
5-MIAA	1.63 ± 0.21	1.23 ± 0.68	4.06 ± 1.30
5-MTOH	0.07 ± 0.02	0.03 ± 0.01	0.11 ± 0.03[b]
NAS	0.02 ± 0.01	1.90 ± 0.53[a]	0.51 ± 0.10[b]
Melatonin	0.12 ± 0.03	1.61 ± 0.30[a]	1.48 ± 0.32

All values represent the means ± SEM of four to six sheep. Pineals from "day" (1730 hours; untreated), "night" (1830 hours; vehicle-treated), and "night + cycloheximide" (1830 hours; cycloheximide 200 mg i.v.) sheep were obtained as described in Materials and Methods. The pineal indoles were measured using HPLC-EC as described in the text.

[a] Significantly different from "day" group ($p < 0.05$).

[b] Significantly different from "night" group ($p < 0.05$).

are not decreased when 5-HT availability is not significantly reduced early in the dark period is consistent with the above proposal.

Our observation that cycloheximide treatment, which completely inhibited the rise in NAT, failed to prevent the rise in serum and pineal melatonin is surprising and does not fit the model of regulation of melatonin synthesis based on rat studies. In this model, the nocturnal rise in melatonin synthesis is primarily a result of a large increase in the activity of NAT (Klein et al., 1981). Perhaps the increase in melatonin in our experiments may be due to an increase in HIOMT activity. The finding that cycloheximide treatment tended to increase HIOMT activity at night and increased pineal 5-MTOH and 5-MIAA is consistent with this proposal, since the hydroxyindoles 5-HTOH and 5-HIAA are substrates of this enzyme.

The results presented in this report in part support the proposal that rapid changes in NAT activity are a factor in the regulation of ovine pineal melatonin synthesis, because the rapid nighttime increase in pineal melatonin is associated with an equally rapid increase in NAT activity, as measured in homogenates, and in NAS. The increase in NAS enhances melatonin synthesis by mass action. The smaller magnitude of NAT response in sheep compared to the rat (Foldes et al., 1984; Namboodiri et al., 1985), makes it reasonable to think that another mechanism, independent of NAT activity, is also involved in the nocturnal increase in pineal melatonin in this species. This hypothesis is supported by our observations that the increase in pineal melatonin following a light pulse at night is not accompanied by a change in NAT activity (Namboodiri et al., 1985) and that the α_1-adrenoceptor blocker, prazosin, inhibits the nighttime increase in ovine serum and pineal melatonin, without affecting NAT activity (Sugden et al., 1985). Further studies are required to identify the NAT-independent mechanism and to understand the relative contribution of this mechanism and NAT to the regulation of pineal melatonin synthesis in sheep. In addition, it will also be necessary to determine if 5-HT acetylation in intact sheep pineal cells is or is not reliably reflected by NAT assays in homogenates as discussed previously (Namboodiri et al., 1985).

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